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Modes of annexin-membrane interactions analyzed by employing chimeric annexin proteins

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Abstract

Annexin II is a member of the annexin family of Ca^{2+}- and phospholipid-binding proteins which is particularly enriched on early endosomal membranes and has been implicated in participating in endocytic events. In contrast to other endosomal annexins the association of annexin II with its target membrane can occur in the absence of Ca^{2+} in a manner depending on the unique N-terminal domain of the protein. However, endosome binding of annexin II does not require formation of a protein complex with the intracellular ligand S100A10 (p11) as an annexin II mutant protein (PM AnxII) incapable of interacting with p11 is still present on endosomal membranes. Fusion of the N-terminal sequence of this PM AnxII (residues 1–27) to the conserved protein core of annexin I transfers the capability of Ca^{2+}-independent membrane binding to the otherwise Ca^{2+}-sensitive annexin I. These results underscore the importance of the N-terminal sequence of annexin II for the Ca^{2+}-independent endosome association and argue for a direct interaction of this sequence with an endosomal membrane receptor.

Keywords: Calcium; Cholesterol; Endocytosis; Membrane organization; Membrane raft

1. Introduction

Members of the annexin family of Ca^{2+}-binding proteins are typically characterized by their Ca^{2+}-regulated interaction with acidic phospholipids and cellular membranes (for review see [1,2]). Binding of the common annexin ligands (Ca^{2+}, phospholipids) is mediated through a structurally conserved domain within the individual proteins, the protein core. Herein, segments of internal sequence homology, the so-called annexin repeats, form type II and type III Ca^{2+}-binding sites and Ca^{2+} bound within these sites serves a bridging function when annexins interact peripherally with membrane phospholipids [3–5]. The second principal domain within an annexin molecule is formed by the N-terminal sequence which precedes the protein core and varies in length and sequence between different members. It is thought to confer functional specificity to the individual annexins and harbors binding sites for protein ligands and sites for different posttranslational modifications (for review see [2]). Functions as diverse as a participation in membrane trafficking and the regulation of blood coagulation have been proposed for different annexins. While these proposals still await final proof in in vivo systems almost all relate to the membrane binding properties of the annexins and it appears that the conserved annexin protein core is a module

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targeting annexins to membrane phospholipids for specific actions.

At least two members of the family, annexins I and II, are present on endosomal membranes and a participation in endocytosis and/or the organization of the endosomal membrane system has been inferred from these localizations and from functional experiments using in vitro systems. Annexin I is a substrate for the tyrosine kinase of the epidermal growth factor (EGF) receptor with phosphorylation most likely occurring in multivesicular endosomes. This phosphorylation converts a Ca^{2+}-independently associated form of annexin I into a species requiring Ca^{2+} for membrane association and it has been proposed that this event is functionally linked to the inward vesiculation in multivesicular endosomes and thus the sorting of internalized receptors destined for degradation [6]. However, the majority of annexin I, at least in BHK [7] and HeLa cells (U. Rescher and V. Gerke, unpublished observation), is found associated with early endosomal membranes and this association requires Ca^{2+}. In contrast to this Ca^{2+}-sensitive and thus typical annexin-membrane interaction a large fraction of the endosome associated annexin II remains bound to the organelle membrane in the presence of Ca^{2+}-chelating agents. Moreover, an annexin II mutant protein with inactivated type II Ca^{2+}-binding sites which is not able to interact with membranes under physiological Ca^{2+} levels binds endosomal membranes when expressed transiently in BHK cells. This binding depends on the presence of the unique N-terminal domain of the protein as it is lost in a mutant protein lacking the first 24 residues [8]. Most likely the atypical, i.e. Ca^{2+}-independent, annexin-membrane association requires a certain membrane composition and configuration as membrane bound annexin II is released specifically together with a group of actin-binding proteins by sequestration of membrane cholesterol [9].

Within most cells the major fraction of annexin II is found in a tight heterotetrameric complex together with the S100 protein S100A10 (p11). Complex formation is highly specific and is also mediated through the unique N-terminal domain of annexin II with residues 1–14 representing the entire S100A10 binding site [10,11]. Although S100A10 is also found on endosomal membranes (D. Zeuschner and V. Gerke, unpublished observation) S100A10 binding is not required for the association of annexin II with endosomes [8].

In addition to the biochemical and morphological evidence there is also functional support for a role of annexin II in endocytosis. The protein is transferred efficiently from a donor to an acceptor membrane in homotypic endosome fusion assays [12] and perturbation of its intracellular localization through ectopic expression of a dominantly interfering annexin II mutant results in a simultaneous perturbation of the early endosome distribution in polarized MDCK cells [13]. It has thus been postulated that annexin II participates in organizing the early endosomal membrane system possibly by linking certain domains of endosomal membranes to an underlying actin cytoskeleton.

Since such a function is tightly linked to the Ca^{2+}-independent membrane association of annexin II we analyzed whether this atypical annexin property can be transferred to the Ca^{2+}-sensitive annexin I. By transiently expressing mutant proteins in BHK cells and by elucidating their intracellular distribution through subcellular fractionation we show that a sequence corresponding to residues 1–27 of annexin II with an inactivated p11 binding site when fused to an annexin I core can transfer the Ca^{2+}-independent membrane binding. This leads to a model in which different, Ca^{2+}-independent and Ca^{2+}-sensitive, membrane interaction can regulate the association of annexin II with endosomes.

2. Material and methods

2.1. Expression constructs

The wild-type and mutant annexin I and annexin II expression constructs have been described [7,8,14]. For generation of the AnxII-AnxI chimera the N-terminal domain of PM annexin II (nucleotides 39–133, amino acid residues 1–27) was amplified by PCR using the vector pCMV-PManxII [8] as template. The vector pCMV-AnxI [7] containing the porcine annexin I cDNA was used for PCR amplification of the annexin I core domain (nucleotides 90–1082, amino acid residues 37–346). PCR primers were designed to generate a unique *Bam*HI site at

the 3′ end of the PMAnxII and the 5′ end of the AnxI core product, respectively. Following appropriate restriction digest the two annexin fragments were ligated together generating a fusion of the N-terminal PM annexin II encoding sequence in frame with that of the annexin I core. The chimeric annexin construct was subcloned into the eukaryotic expression vector pCMV5 to yield the pCMV/AnxII-$_{PM1-27}$AnxI construct which was used for transfection of BHK-P3 cells.

2.2. Transfection of BHK-P3 cells

BHK-P3 cells (confluent 60 cm^2 culture dish) were washed once in cold PBS and resuspended in 400 μl PBS. 200 μl of the cell suspension were mixed with 10 μg plasmid DNA. Electroporation was carried out at 900 μF, 250 V using a Gene pulser II (Bio-Rad). After a 10 min incubation on ice the cells were resuspended in warm DMEM and seeded in appropriate density.

2.3. Fractionation of endosomal membranes from BHK-P3 cells

The fractionation of endosomal membranes employed sucrose step gradients and was carried out as described [15,16]. Membranes contained in the isolated fractions were pelleted by ultracentrifugation (150 000 × g, 15 min, 4°C) and analyzed by SDS-PAGE and Western blotting. Immunoblotting employed antibodies specifically recognizing the ectopically expressed and not the endogenous annexin proteins [7,14].

2.4. Membrane extractions

The Ca^{2+} dependence of the annexin II-membrane association was analyzed by collecting endosomal membranes from a sucrose gradient prepared in the presence and absence of 1 mM EDTA, respectively. The isolated membranes were analyzed by SDS-PAGE and Western blotting using the specific antibodies mentioned above.

The effect of cholesterol on the membrane binding of annexin II was investigated using membranes from the early endosomal fraction which had been isolated in the presence of EDTA. The early endosomal membranes were washed once in HBSE (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EGTA) and the membrane pellets were then resuspended in HBSE containing 0,5 mg/ml digitonin and incubated for 30 min at room temperature. Extracted membranes were collected by centrifugation (150 000 × g, 15 min, 4°C) and suspended in SDS-sample buffer. The proteins released by digitonin from the membranes into the supernatant were precipitated using TCA and then dissolved in SDS-sample buffer. All samples were analyzed by SDS-PAGE and Western blotting employing the monoclonal anti-annexin II antibody HH7 [14] for detection of the endogenous BHK protein.

For characterization of the binding characteristics of mutant annexin II or chimeric annexin II-annexin I proteins, BHK-P3 cells were transfected transiently with the relevant constructs two days before the extraction experiments. Fractionation and extraction were then performed as described above.

3. Results and discussion

Previous work employing mutant annexin II proteins ectopically expressed in BHK cells had revealed that the N-terminal sequence of residues 1–27 is indispensable for the unique Ca^{2+}-independent membrane binding displayed by this protein. Moreover, mutations within this sequence which inactivate S100A10 binding, the so-called p11-minus (PM) substitutions I6E and L7E, do not interfere with the Ca^{2+}-independent endosome binding [8]. To analyze whether this atypical annexin property is contained within the unique N-terminal annexin II sequence and can be transferred to another annexin whose membrane binding is Ca^{2+}-sensitive we generated the chimeric AnxII$_{PM1-27}$AnxI protein. Here residues 1–27 of the PM annexin II sequence, i.e. the N-terminal sequence incapable of binding S100A10, were fused to the annexin I core domain thereby replacing the unique N-terminal domain of annexin I (Fig. 1).

In parallel experiments the chimeric annexin protein AnxII$_{PM1-27}$AnxI as well as its parental molecules PM AnxII and AnxI were expressed transiently in BHK cells. Subsequently, their distribution over endosomal membranes was analyzed by fractionation of such membranes in a sucrose flotation gradient

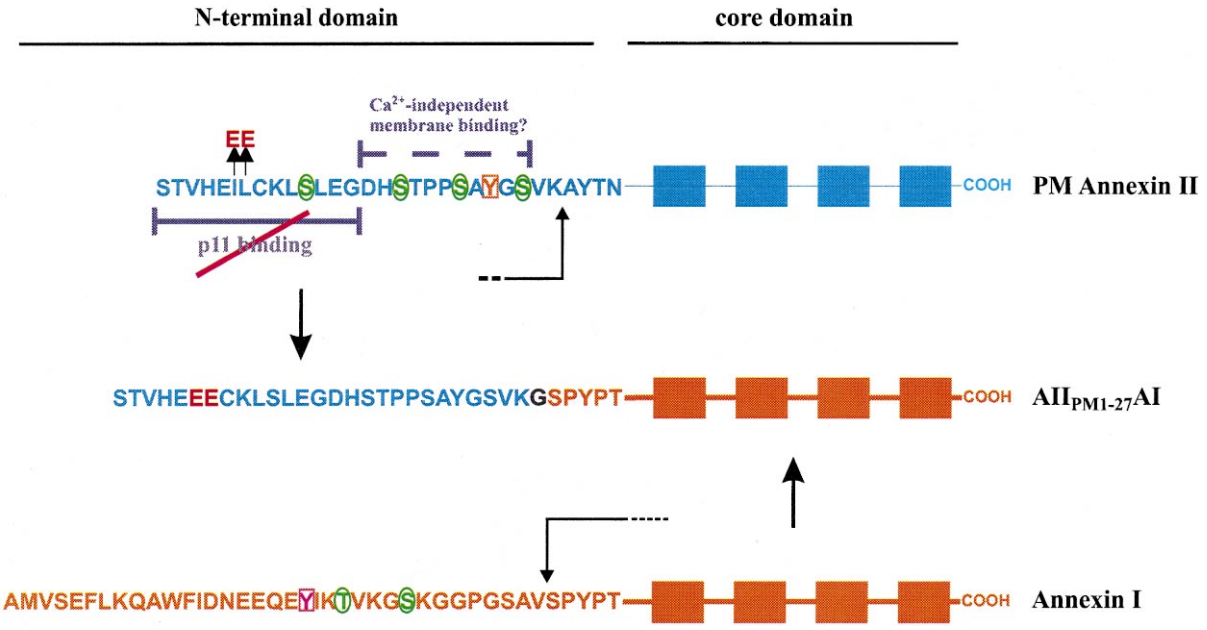

Fig. 1. Construction of the $AnxII_{PM1-27}AnxI$ chimera. Annexin structures are shown schematically with the conserved protein core depicted as four rectangles representing the four annexin repeats. Sequences of the N-terminal domains are given in the one letter code. As compared to the wild-type sequence of the human protein PM annexin II contains two amino acid substitutions (I6E and L7E) which render the S100A10 (p11) binding site non-functional. Residues 1–27 of this PM annexin II were placed in front of an annexin I core domain (amino acids 37–346) to generate the $AnxII_{PM1-27}AnxI$ chimera ($AII_{PM1-27}AI$). Residues phosphorylated in vitro by serine/threonine- or tyrosine-specific protein kinases are given in green and red, respectively.

and immunoblotting of the fractions obtained with antibodies specifically recognizing the ectopically expressed proteins and not the endogenous annexins. Fig. 2 shows such comparative immunoblots of endosomal membrane fractions prepared either in the presence (−EDTA) or the absence (+EDTA) of micromolar Ca^{2+} concentrations. As observed previously [7,8], endogenous (not shown) and ectopically expressed annexin II (AnxII) remain associated with early endosomal membranes in the presence of a Ca^{2+} chelator whereas annexin I (AnxI) requires Ca^{2+} for its interaction with early endosomes. Ca^{2+}-independent endosome binding is also displayed by the PM AnxII derivative incapable of binding S100A10. Thus complex formation with endogenous S100A10 is not a prerequisite for the endosome-annexin II interaction occurring in the absence of Ca^{2+}. However, when compared to the isolation of endosomes carried out in the absence of EDTA the fraction of early endosome-associated PM annexin II is considerably reduced in the absence of Ca^{2+}. This is not due to an alteration of the fractionation properties of early endosomes as a typical marker of this compartment, the early endosome associated antigen EEA1 [17], is unaffected by the EDTA treatment. Thus it appears that the Ca^{2+}-independent binding of annexin II to early endosomes is at least in part facilitated or stabilized by formation of the annexin II-S100A10 complex.

Even though displaying a reduced membrane binding in the absence of Ca^{2+} PM annexin II still behaves different when compared to annexin I which shows no detectable membrane association in the presence of EDTA. However, the unique properties of PM annexin II can be transferred to the annexin I protein when the N-terminal annexin I domain is replaced by the N-terminal PMAnxII sequence in the $AnxII_{PM1-27}AnxI$ chimera (Fig. 2). Thus, when fused to an annexin core the N-terminal annexin II sequence covering residues 1–27 is sufficient for mediating the unique property of Ca^{2+}-independent membrane association. This argues for the existence of a receptor structure on endosomal membranes which specifically interacts with the annexin II sequence contained within the N-terminal 27 residues of PM annexin II. As the Ca^{2+}-independent binding

is not observed for annexin II when artificial phospholipid bilayers are employed in liposome pelleting assays at physiological pH [18] such receptor structure is specific for cellular membranes. Collectively these observations point towards the existence of a specific endosomal receptor (lipid membrane structure or membrane associated/bound protein) which recognizes the N-terminal sequence of annexin II.

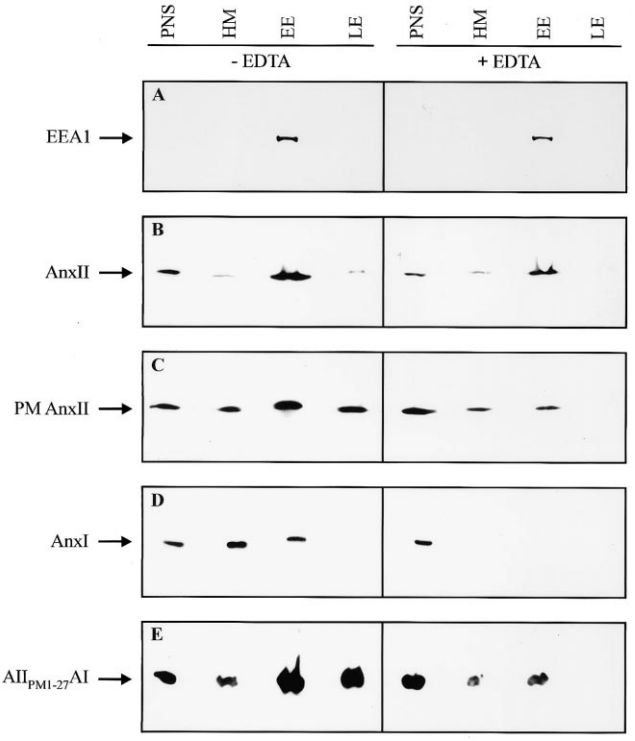

Fig. 2. Association of ectopically expressed wild-type and mutant annexin proteins with membrane fractions from BHK-P3 cells. Wild-type annexin II (AnxII), wild-type annexin I (AnxI), the PM annexin II mutant (PM AnxII) or the AnxII$_{PM1-27}$AnxI chimera was ectopically expressed in BHK-P3 cells. Two days following transfection membranes present in a postnuclear supernatant (PNS) of the lysed cells were subjected to separation in a flotation gradient. Cell lysis and gradient fractionation were carried out either in the presence (+) or the absence (−) of 1 mM EDTA. Gradient fractions enriched in heavy membranes (HM), early endosomes (EE) or late endosomes (LE) were analyzed for the presence of the different annexin derivatives by immunoblotting with antibodies specifically recognizing the ectopically expressed proteins. Immunoblots with antibodies against the early endosomal protein EEA1 served as a control to verify the enrichment of early endosomes in the EE fraction in the different experiments (only one such control is shown). Note that the AnxII$_{PM1-27}$AnxI chimera shows a distribution among the membrane fractions which is similar to that of PM AnxII. In contrast, AnxI fails to co-fractionate with cellular membranes in the presence of EDTA.

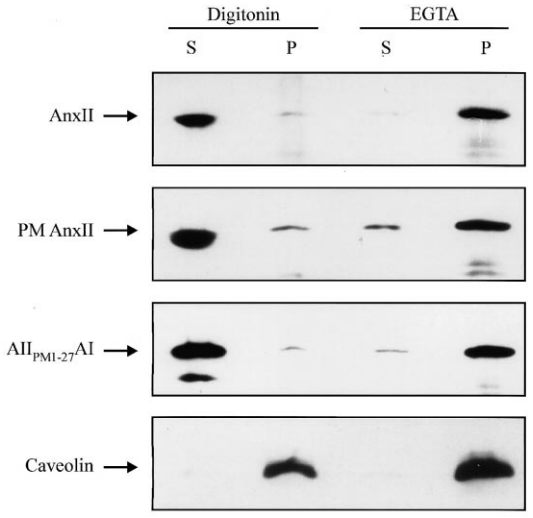

Fig. 3. Release of membrane bound annexin II and different mutant annexin II derivatives by digitonin. PM annexin II or the AnxII$_{PM1-27}$AnxI chimera was expressed transiently in BHK-P3 and endosomal membranes of the transfected cells were subjected to flotation gradient separation in the presence of EDTA. Membranes collected from the early endosomal fraction were treated with digitonin- or EGTA-containing buffers and centrifuged again to separate the remaining membranes (P) from the proteins released into the supernatant (S). Immunoblotting using specific antibodies revealed the presence of endogenous annexin II (AnxII) as well as the ectopically expressed PM annexin II (PM AnxII) and AnxII$_{PM1-27}$AnxI (AII-$_{PM1-27}$AI) derivatives in the different fractions. Note that endogenous annexin II, PM annexin II and the AnxII$_{PM1-27}$AnxI chimera are extracted almost quantitatively from the early endosomal membranes by digitonin. In contrast, membrane bound caveolin is resistant to extraction under these conditions.

Ca^{2+}-independent binding of annexin II to cellular membranes requires a certain lipid composition and/or structure with membrane cholesterol being of prime importance [9]. To analyze whether this property is also mediated through the N-terminal PMAnxII sequence we subjected early endosomal membranes prepared from BHK cells transiently expressing different mutant annexins to extraction with the cholesterol-binding drug digitonin. While the pool of endogenous (not shown) and ectopically expressed annexin II associated with the endosome fraction in the absence of Ca^{2+} remains bound upon further wash with EGTA-containing buffers it is almost quantitatively extracted by the digitonin treatment (Fig. 3). The endosome bound PM annexin II

behaves identically while membrane associated caveolin is resistant to digitonin treatment under the conditions chosen. Again the unique property of annexin II and its PM derivative is contained within the N-terminal sequence as the endosome associated fraction of $AnxII_{PM1-27}AnxI$ shows the same digitonin sensitivity (Fig. 3). Thus, the Ca^{2+}-independent association of annexin II with endosomal membranes is mediated through the N-terminal 27 residues which interact with receptor structures found within domains rich in or stabilized through membrane cholesterol. Such an association of annexin II with cholesterol-rich membrane domains (or protein receptors therein) is in line with the finding that the Ca^{2+}-independently associated pool of endosome bound annexin II is resistant to Triton X-100 treatment at 4°C (data not shown) thus showing a behavior typical of components of sphingolipid- and cholesterol-rich membrane rafts [19,20]. The binding of annexin II (and the $AnxII_{PM1-27}AnxI$ chimera) to cholesterol-rich domains of endosomal membranes is different to what has been observed for plasma membrane associated annexin II. While the former can occur in the absence of Ca^{2+} the latter requires Ca^{2+} and

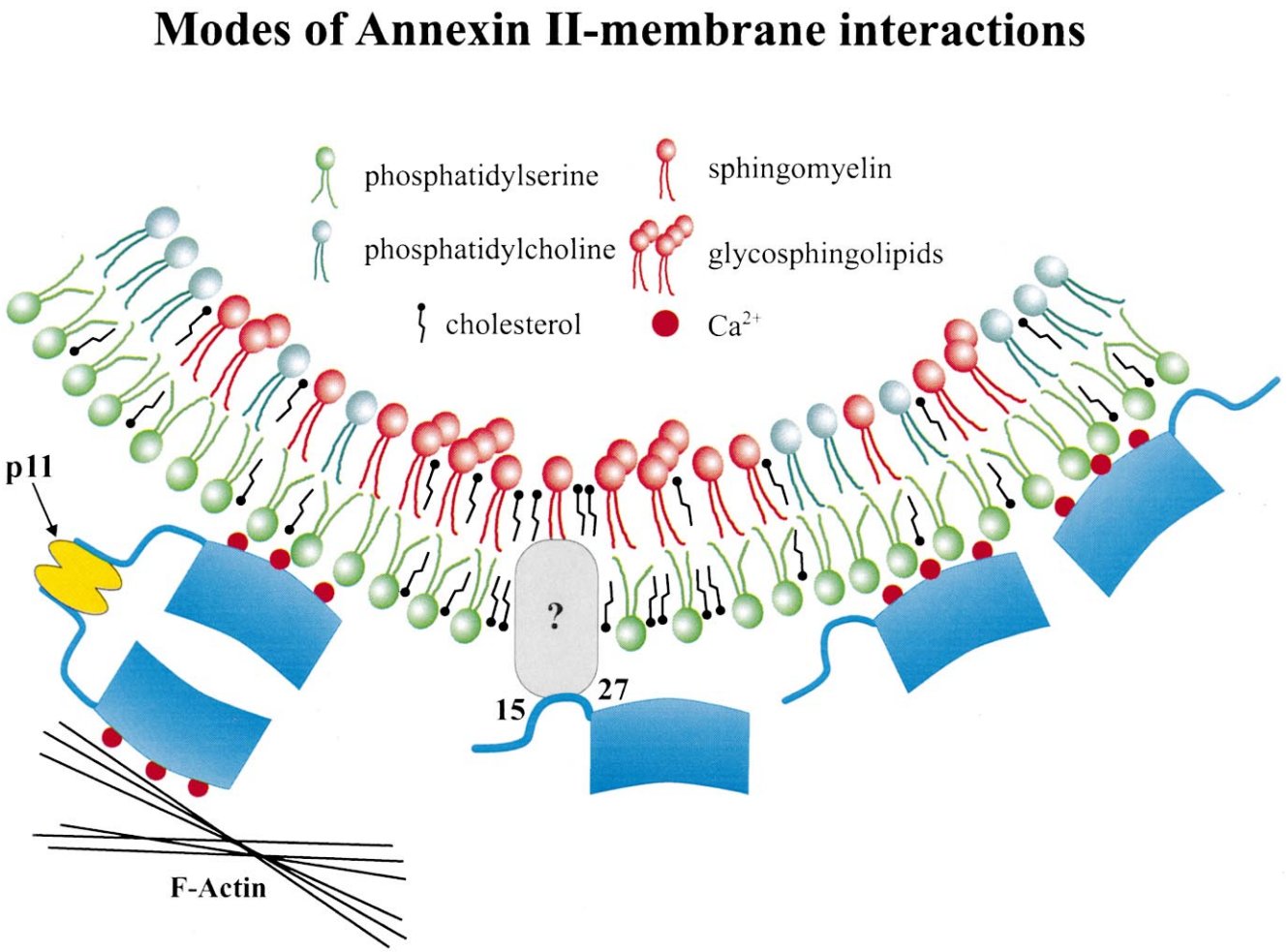

Fig. 4. Schematic model depicting different modes of annexin II-membrane interactions. Monomeric annexin II (blue) and the heterotetrameric annexin II-p11 complex can bind to negatively charged phospholipid head groups in a peripheral and Ca^{2+}-regulated manner with the divalent cations serving a bridging function. Endosomal membranes contain a receptor structure (?) most likely within cholesterol-rich domains which can interact with the N-terminal domain of annexin II in a Ca^{2+}-independent manner. It appears that a unique sequence contained within the first 27 residues of annexin II but different from the S100A10 (p11) binding site, i.e. amino acids 15–27, represents the binding site for the endosomal receptor.

thus most likely an additional interaction of the annexin protein core with negatively charged phospholipids [21,22].

Collectively, the results obtained with mutant and chimeric annexin proteins point towards different modes of interaction of annexin II with endosomal membranes which are regulated differently by Ca^{2+}. As depicted schematically in Fig. 4, both the annexin II monomer and the annexin II-S100A10 complex can bind peripherally and Ca^{2+} dependently to negatively charged membrane phospholipids with the latter – due to the F-actin binding of annexin II [23,24] – having the capability of linking the membrane surface to underlying actin filaments. Alternatively or additionally the N-terminal sequence of the protein can interact with a receptor structure in the absence of Ca^{2+}. The functional integrity of the receptor structure as an annexin II-binding platform requires membrane cholesterol but does not depend on S100A10 binding. Similar modes of action are also likely to occur at the plasma membrane although here the association with cholesterol-rich membrane domains (or a receptor therein) requires an additional Ca^{2+}-dependent interaction. Thus it appears that a function of annexin II at its target membrane is strictly dependent on Ca^{2+} binding to the protein only in the case of the plasma membrane.

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References

[1] P. Raynal, H.B. Pollard, Biochim. Biophys. Acta 1197 (1994) 63–93.
[2] V. Gerke, S.E. Moss, Biochim. Biophys. Acta 1357 (1997) 129–154.
[3] R. Huber, M. Schneider, I. Mayr, J. Römisch, E.-P. Paques, FEBS Lett. 275 (1990) 15–21.
[4] X. Weng, H. Luecke, I.S. Song, D.S. Kang, S.H. Kim, R. Huber, Protein Sci. 2 (1993) 448–458.
[5] M.A. Swairjo, N.O. Concha, M.A. Kaetzel, J.R. Dedman, B.A. Seaton, Nat. Struct. Biol. 2 (1995) 968–974.
[6] C.E. Futter, S. Felder, J. Schlessinger, A. Ullrich, C.R. Hopkins, J. Cell Biol. 120 (1993) 77–83.
[7] J. Seemann, K. Weber, M. Osborn, R.G. Parton, V. Gerke, Mol. Biol. Cell 7 (1996) 1359–1374.
[8] M. Jost, D. Zeuschner, J. Seemann, K. Weber, V. Gerke, J. Cell Sci. 110 (1997) 221–228.
[9] T. Harder, R. Kellner, R.G. Parton, J. Gruenberg, Mol. Biol. Cell 8 (1997) 533–545.
[10] N. Johnsson, G. Marriott, K. Weber, EMBO J. 7 (1988) 2435–3442.
[11] S. Rety, J. Sopkova, M. Renouard, D. Osterloh, S. Tabaries, F. Russo-Marie, A. Lewit-Bentley, Nat. Struct. Biol. 6 (1999) 89–95.
[12] N. Emans, J.P. Gorvel, C. Walter, V. Gerke, R. Kellner, G. Griffiths, J. Gruenberg, J. Cell Biol. 120 (1993) 1357–1369.
[13] T. Harder, V. Gerke, J. Cell Biol. 123 (1993) 1119–1132.
[14] C. Thiel, M. Osborn, V. Gerke, J. Cell Sci. 103 (1992) 733–742.
[15] J.P. Gorvel, P. Chavrier, M. Zerial, J. Gruenberg, Cell 64 (1991) 915–925.
[16] F. Aniento, F. Gu, R.G. Parton, J. Gruenberg, J. Cell Biol. 133 (1996) 29–41.
[17] A. Simonsen, R. Lippe, S. Christoforidis, J.M. Gaullier, A. Brech, J. Callaghan, B.H. Toh, C. Murphy, M. Zerial, H. Stenmark, Nature 394 (1998) 494–498.
[18] J. König, J. Prenen, B. Nilius, V. Gerke, J. Biol. Chem. 273 (1998) 19679–19684.
[19] R.G. Parton, K. Simons, Science 269 (1995) 1398–1399.
[20] T. Harder, K. Simons, Curr. Opin. Cell Biol. 9 (1997) 534–542.
[21] T. Harder, V. Gerke, Biochim. Biophys. Acta 1223 (1994) 375–382.
[22] S. Oliferenko, K. Paiha, T. Harder, V. Gerke, C. Schwarzler, H. Schwarz, H. Beug, U. Günthert, L. Huber, J. Cell Biol. 146 (1999) 843–854.
[23] V. Gerke, K. Weber, EMBO J. 3 (1984) 227–233.
[24] P.G. Jones, G.J. Moore, D.M. Waisman, J. Biol. Chem. 267 (1992) 13993–13997.